MOLECULAR PHARMACOLOGY, 8, 41–49

Relative Binding Sites of Pharmacologically Active Ligands on Bovine Erythrocyte Acetylcholinesterase

B. D. ROUFOGALIS AND E. E. QUIST

Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver 8, British Columbia, Canada

(Received August 9, 1971)

SUMMARY

The kinetics of the interaction of partially purified bovine erythrocyte acetylcholinesterase (EC 3.1.1.7) with calcium, tetramethylammonium, tetraethylammonium, decamethonium, gallamine, and *d*-tubocurarine has been investigated, using acetylcholine as substrate. Antagonism between various combinations of ligands has been studied. Decamethonium binds to the catalytic anionic site (α) and to an allosteric site (β). Calcium (0.2 mM) competes with decamethonium but not with acetylcholine, and is considered to act at the β-anionic site. This is an accelerator site, which may bind tetraethylammonium and possibly other polar cations. Tetraethylammonium may also bind to the catalytic site, α. Tetramethylammonium, which is not an accelerator, is considered to bind to the catalytic site exclusively. Neither tetramethylammonium, tetraethylammonium, nor calcium antagonizes the binding of gallamine. This observation, together with that of the partially competitive nature of the inhibition by gallamine, indicates that gallamine cannot bind to the α- or β-anionic sites and hence must bind to a second allosteric site, γ. The ability of gallamine to antagonize inhibition by decamethonium is attributed to allosteric perturbations of the α- and β-sites induced by the action of gallamine at the γ-site.

INTRODUCTION

Recently significant evidence has accumulated indicating that acetylcholinesterase (EC 3.1.1.7) possesses, in addition to an anionic site in the catalytic center, peripheral anionic sites where ligands bind and exert a regulatory role on the enzyme activity. Evidence for the presence of such allosteric sites was first presented by Changeux (1), from experiments with neuromuscular blocking agents of the depolarizing and nondepolarizing types. Decamethonium, a depolarizing blocker with two cationic groups, has a high affinity for acetylcholinesterase (0.01 μM) at low ionic strength. It presumably acts in bivalent fashion with the enzyme (1). The inhibition by decamethonium follows Michaelis-Menten kinetics, and it is considered that one of the trimethylammonium groups binds to the catalytic anionic site and the other to a peripheral anionic site (1–3). On the other hand, inhibition of the enzyme by the nondepolarizing neuromuscular blockers gallamine and *d*-tubocurarine does not follow the laws of simple competitive inhibition but obeys partially competitive kinetics (1, 2). Such inhibition implies that the substrate and effector are bound simultaneously by the enzyme (4), suggesting that these neuromuscular blockers are bound at peripheral anionic sites (1).

This research was supported by the Medical Research Council of Canada and (in the latter part) by the Defense Research Board of Canada through Grant 9370-14.

In addition to neuromuscular blockers, acetylcholinesterase recognizes, in rather specific ways, various small quaternary ammonium ions and inorganic ions. It has been shown (5–7) that some such ions can accelerate deacylation reactions of acetylcholinesterase whereas related analogues cannot. It has been suggested that accelerators and nonaccelerators may bind and exert their actions at different sites on the enzyme (8, 9), but definitive evidence has so far been lacking. In addition, it has been suggested that inorganic ions may bind at allosteric sites on acetylcholinesterase (1, 10–12).

We report here on kinetic and antagonism studies of the relationship between the binding sites for small quaternary ammonium ions (tetramethyl- and tetraethylammonium), substrate, neuromuscular blockers, and calcium with a purified bovine erythrocyte preparation. An expanded binding-site model is presented.

MATERIALS AND METHODS

Enzyme. Acetylcholinesterase was a partially purified bovine erythrocyte preparation (Nutritional Biochemicals Corporation). In the absence of added ions (ionic strength less than 0.001) the enzyme had a specific activity of 150 μmoles of acetylcholine hydrolyzed per hour per milligram of protein, and a K_m value of 27 μM, at a protein concentration of 3.75 μg/ml. Enzyme solutions (7.5 mg/25 ml) were prepared in distilled water every 2 days and maintained at 0–4°.

Assay. Reaction rates were measured with a Radiometer pH-Stat. Sodium hydroxide (0.01 N) from a syringe burette (0.5 ml) was used to maintain the pH. All rates were measured at 25 ± 0.1°, the final volume of the reaction medium being 40 ml. The solutions were stirred mechanically throughout the assays, and dry nitrogen was passed over the surface. The pH was maintained at 7.4 ± 0.05, corrections being made for deviations due to stirring at low ionic strength as previously described (5). No ions were added to the reaction medium except when the effect of ions was to be studied.

Procedure. To the calculated volume of medium was added enzyme, followed by the ligand under study. After 1 min the mixture was stirred for 5 min under a steady stream of nitrogen. The nitrogen was withdrawn to a level just above the surface, the pH was adjusted to 7.4 if necessary, and the substrate was added to start the reaction. When a wide range of ligand concentrations was studied, the lowest concentration of ligand was first incubated with the enzyme as described above, and the reaction was started by substrate addition. When the velocity became constant, further aliquots of ligand were added sequentially. If the total change in volume was no more than 1.5% and the total change in substrate concentration did not exceed 2%, the results obtained by this method were comparable to those obtained in individual assays. Controls, run at the beginning and end of each series of experiments, were constant to within ±3.5%. When large quaternary ammonium compounds were used, strict precautions for cleaning the glass vessels, the polyethylene parts of the reaction vessel, and the electrodes were found to be critical for accurate results.

Chemicals. The following compounds were used as received: tetramethylammonium iodide, tetraethylammonium iodide, and calcium chloride dihydrate (Baker Chemical Company); decamethonium bromide (K & K Laboratories, Inc.); and acetylcholine perchlorate (British Drug Houses). Gallamine triethiodide was a gift from Poulenc, Ltd.

RESULTS

Kinetics of Ligand Interactions

Kinetic data were obtained, relative to controls at ionic strength less than 0.001, using acetylcholine as substrate.

Substrate inhibition. In the absence of added ions the substrate dependence data gave an asymmetrical, bell-shaped curve, with an optimum substrate concentration of 0.4–0.5 mM.

Calcium. Eadie plots (Fig. 1) show that Ca^{++} (0.2 mM) did not alter the K_m of acetylcholine for the enzyme (Table 1) and did not compete with acetylcholine for the catalytic anionic site. This suggests that Ca^{++} can bind to the enzyme at a peripheral anionic site, simultaneously with the bound substrate.

Neuromuscular blockers. The Eadie plots

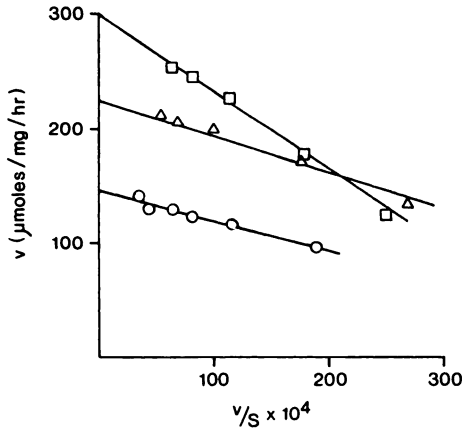

FIG. 1. *Effect of calcium on acetylcholine hydrolysis*
The control plot (○——○) is the average of a number of determinations in the absence of added ions (see the text). Substrate concentrations were varied from 0.05 to 0.4 mM. △——△, 0.2 mM Ca^{++}; □——□, 1 mM Ca^{++}.

TABLE 1

Effects of some compounds on kinetics of acetylcholine hydrolysis

V_{max} is the maximum hydrolysis for each compound, and is expressed as micromoles of acetylcholine hydrolyzed per milligram of protein per hour. K_i values were obtained from Dixon plots (4) at two or three substrate concentrations. $K_{m(app)}$ is the negative slope of Eadie plots (Fig. 1), at various substrate concentrations, in the presence or absence of compound. Control values were obtained in the absence of added ions or compounds.

Compound	V_{max}	$K_{m(app)}$	K_i
		M	*M*
Control	146	2.7×10^{-5}	
Decamethonium	100		4.6×10^{-8}
d-Tubocurarine	110		1.9×10^{-5}
Gallamine	228		1.8×10^{-5}
TEA	202		
TMA	144		
$CaCl_2$ (0.2 mM)	225	3.1×10^{-5}	

in Fig. 2 show that decamethonium and *d*-tubocurarine gave mixed competitive-noncompetitive inhibition. The maximum velocity (V_{max}) in the presence of decamethonium (0.15 μM) was reduced from 146 μmoles/mg/hr for the control to 100, and for *d*-tubocurarine (20 μM), to 110 (Table 1).

Figure 2 shows that gallamine was not an inhibitor at all substrate concentrations; at high substrate concentrations the reaction was accelerated and V_{max} in the presence of gallamine was increased from 146 to 228 μmoles/mg/hr. Gallamine accelerated the decarbamylation reaction of acetylcholinesterase (3); hence the observed increase in V_{max} could have been due to acceleration of the deacetylation step in the hydrolysis sequence.

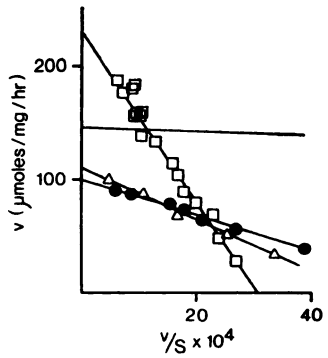

FIG. 2. *Effects of gallamine, d-tubocurarine, and decamethonium on acetylcholine hydrolysis.*
The control plot (experimental points not shown) is the same as that in Fig. 1. Substrate concentrations were varied from 0.1 to 3 mM. □——□, 20 μM gallamine; △——△, 20 μM *d*-tubocurarine; ●——●, 0.15 μM decamethonium.

TMA and TEA.[1] Table 1 shows that TEA (2 mM) increased the maximum velocity of acetylcholine hydrolysis from 146 to 202 μmoles/mg/hr, whereas TMA (2 mM) had no effect on this quantity.

Estimation of kinetic constants. Apparent Michaelis-Menten constants [$K_{m(app)}$] and maximum velocities (V_{max}) were obtained from Eadie plots as previously described (5), and K_i values were obtained from Dixon plots (4) at two or three substrate concentrations. Representative plots are shown in Fig. 3. Decamethonium obeyed Michaelis-Menten kinetics up to at least 80% inhibition, whereas curvature in the plots for gallamine and *d*-tubocurarine is evident after less than 50% inhibition. The curvature of the gallamine and *d*-tubocurarine plots indicates partially competitive kinetics for these compounds, in agreement with results obtained on the enzyme from electric eel (1). The results are summarized in Table 1.

[1] The abbreviations used are: TMA, tetramethylammonium iodide; TEA, tetraethylammonium iodide.

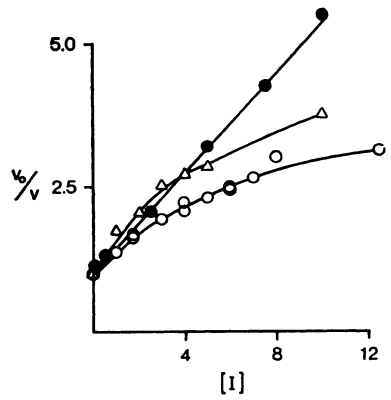

FIG. 3. *Effects of various concentrations of gallamine, d-tubocurarine, and decamethonium on acetylcholinesterase activity.*

The inhibitor concentration was varied at a constant acetylcholine concentration: 0.4 mM in the presence of gallamine and *d*-tubocurarine, and 0.5 mM in the presence of decamethonium. [*I*] is the concentration of each inhibitor, in the following multiples: ●——●, decamethonium ($\times 10^{-7}$ M); ○——○, gallamine ($\times 10^{-5}$ M); △——△, *d*-tubocurarine ($\times 10^{-5}$ M).

Antagonism Studies

The effects of Ca^{++}, TMA, and TEA on the inhibition of acetylcholinesterase by decamethonium, gallamine, and *d*-tubocurarine were studied.

Antagonism by Ca^{++}

Decamethonium. Figure 4A shows curves representing the fractional inhibition by decamethonium (v/v_0) as a function of decamethonium concentration. In the presence of increasing Ca^{++} concentrations, the curves are shifted toward higher decamethonium concentrations.[2] Double-reciprocal plots of the effects of Ca^{++} at various decamethonium concentrations (Fig. 4B) show that the antagonism between decamethonium and Ca^{++} approached competitive kinetics.

Gallamine. Figure 5 shows the fractional inhibition by gallamine (v/v_0) as a function of gallamine concentration. Increasing Ca^{++} concentrations did not cause a shift in the inhibition curves, indicating that Ca^{++} and

[2] This is not an ionic strength effect, since Na^+ and TMA, at concentrations producing the same ionic strength as Ca^{++} (0.2 mM), do not antagonize decamethonium inhibition.

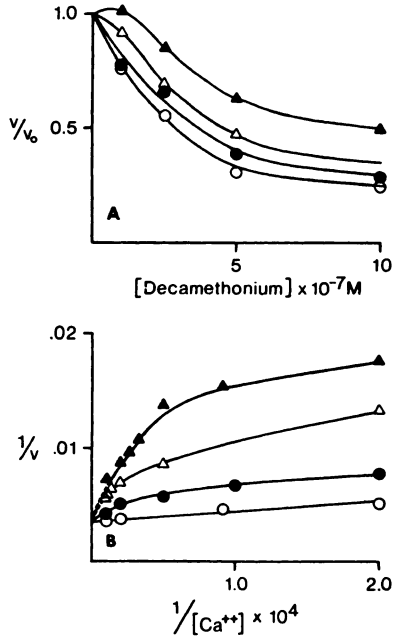

FIG. 4. *Effects of decamethonium and calcium on acetylcholinesterase.*

A. Inhibition by decamethonium at various calcium concentrations. v/v_0 is the fractional inhibition by decamethonium; v is the rate of hydrolysis of acetylcholine in the presence of decamethonium, and v_0 is the rate of hydrolysis of the control at each calcium concentration. The substrate concentration was 0.5 mM. ○——○, control (no calcium); ●——●, 0.05 mM Ca^{++}; △——△, 0.2 mM Ca^{++}; ▲——▲, 1 mM Ca^{++}.

B. Effect of calcium at various concentrations of decamethonium. $1/v$ is the reciprocal of the velocity in micromoles per milligram per hour. The substrate concentration was 0.5 mM. ○——○, control (no decamethonium); ●——●, 0.25 μM decamethonium; △——△, 0.5 μM decamethonium; ▲——▲, 1 μM decamethonium.

gallamine do not compete, and hence must act at different sites.

d-Tubocurarine. Figure 6 shows that Ca^{++} competes with *d*-tubocurarine. The maximum Ca^{++}-induced increase in the activity of the enzyme inhibited by *d*-tubocurarine is 140%, whereas the corresponding increase in the case of decamethonium is 300%. Thus we conclude that the competition by Ca^{++} for *d*-tubocurarine is not complete.

Antagonism by TEA

Decamethonium. Concentrations of TEA which alone affected the control activity less

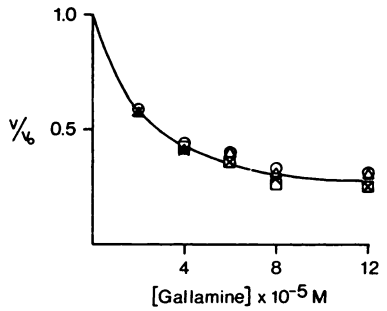

FIG. 5. *Inhibition of acetylcholinesterase by gallamine at various calcium concentrations.*

v/v_0 is the fractional inhibition by gallamine at each calcium concentration. The substrate concentration was 0.4 mM. O——O, control (no Ca^{++}); △——△, 0.1 mM Ca^{++}; ×——×, 0.4 mM Ca^{++}; □——□, 1 mM Ca^{++}.

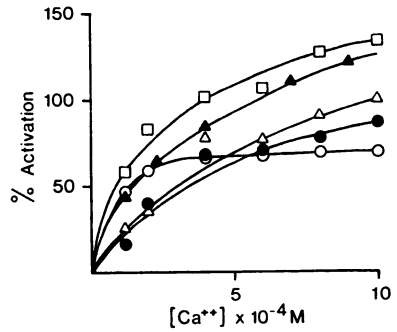

FIG. 6. *Effect of calcium on acetylcholinesterase activity at various concentrations of d-tubocurarine.*

The ordinate represents the percentage increase in the rate of hydrolysis of acetylcholine due to calcium, relative to a control in the presence (or absence) of various concentrations of *d*-tubocurarine. The substrate concentration was 0.4 mM. O——O, control (no *d*-tubocurarine); ▲——▲, 20 μM *d*-tubocurarine; □——□, 40 μM *d*-tubocurarine; △——△, 60 μM *d*-tubocurarine; ●——●, 80 μM *d*-tubocurarine.

than 5% increased the activity of acetylcholinesterase inhibited by decamethonium up to 100% (Fig. 7). A double-reciprocal plot (not shown) indicated that this antagonism approached competitive kinetics. A plot of fractional inhibition (v/v_0) against decamethonium concentration at increasing TEA concentrations showed a shift of the decamethonium inhibition curves to higher concentrations, almost identical with the shift seen with Ca^{++} (see Fig. 4A). Thus it appears that TEA also competes with decamethonium for one or both of its cationic sites.

Gallamine. Increasing TEA concentrations did not antagonize the binding of gallamine, since a plot similar to Fig. 5 was obtained. We therefore conclude that the site for TEA is not the same site occupied by gallamine.

Antagonism by TMA

Decamethonium. Concentrations of TMA which inhibited the enzyme 8% increased its activity when inhibited by decamethonium (Fig. 8). However, comparison of Fig. 8 with Fig. 7 shows that TMA was not as effective as TEA in antagonizing decamethonium inhibition. In the presence of decamethonium (0.5 μM) TMA increased activity by only 43%, whereas the same concentration of TEA increased activity by 100%. The shift in the inhibition curve for decamethonium caused by the presence of TMA (1 mM) is likewise only half that observed with TEA (1 mM). TMA is a pure competitive inhibitor of acetylcholine and hence probably competes with it for the catalytic anionic site. Hence the antagonism of decamethonium by TMA probably occurs at this site.

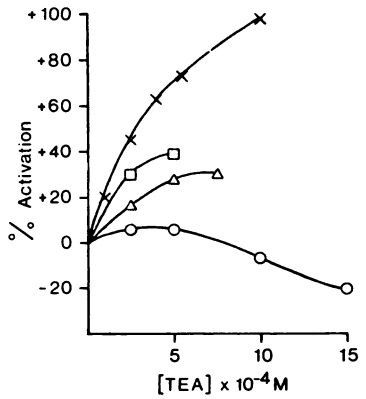

FIG. 7. *Effect of TEA on acetylcholinesterase activity at various concentrations of decamethonium.*

The percentage activation by TEA was obtained as for Ca^{++} in Fig. 6, at the concentrations of decamethonium listed below. The substrate concentration was 0.5 mM. O——O, control (no decamethonium); △——△, 0.1 μM decamethonium; □——□, 0.25 μM decamethonium; ×——×, 0.5 μM decamethonium.

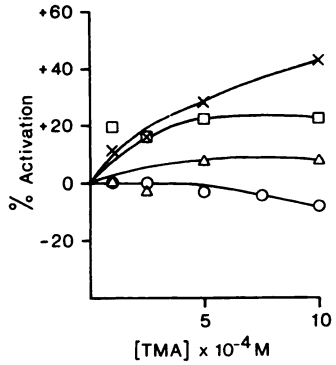

FIG. 8. *Effect of TMA on acetylcholinesterase activity at various concentrations of decamethonium.*

The percentage activation by TMA was obtained as in Fig. 7, at the decamethonium concentrations listed below. The substrate concentration was 0.5 mM. O——O, control (no decamethonium); △——△, 0.1 μM decamethonium; □——□, 0.25 μM decamethonium; ×——×, 0.5 μM decamethonium.

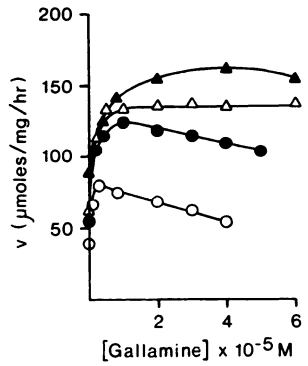

FIG. 9. *Effect of gallamine on acetylcholinesterase activity in the presence of decamethonium at various substrate concentrations.*

v, the velocity of acetylcholine hydrolysis, was obtained at a constant decamethonium concentration (0.5 μM), in the presence of the gallamine concentrations shown on the abscissa. The acetylcholine concentration was varied as follows: O——O, 0.4 mM; ●——●, 1 mM; △——△, 2 mM; ▲——▲, 3 mM.

Gallamine. TMA (0.5 mM) did not antagonize the inhibition of the enzyme by gallamine.

Antagonism of Decamethonium by Gallamine

Figure 9 shows the effect of gallamine on the inhibition of acetylcholinesterase by decamethonium at various substrate concentrations. Gallamine increased the activity of the enzyme inhibited by decamethonium about 100 % at all substrate concentrations studied. The concentration of gallamine that produced the maximum increase in activity (constant decamethonium concentration) was shifted progressively to higher values as the concentration of substrate was increased.

Substrate Inhibition Sites

Substrate inhibition was studied in the presence of Ca^{++}, TEA, and gallamine (Fig. 10). The concentration of substrate at which substrate inhibition appeared was essentially the same as for the control in the presence of Ca^{++}, but TEA and gallamine delayed substrate inhibition. We conclude that the Ca^{++} site is not a site of substrate inhibition.

Antagonism Studies Using $[I]_{50}$ Values

For two inhibitors acting independently (summation),

$$i_{1,2} = i_1 + i_2 - i_1 i_2$$

where $i_{1,2}$ is inhibition in the presence of two inhibitors and i_1 and i_2 represent the effects of the inhibitors separately (4). Combinations of TMA and TEA gave additive effects, as shown in Table 2 (in some cases there is a small amount of synergism). Thus we conclude that TEA and TMA act at two independent sites.

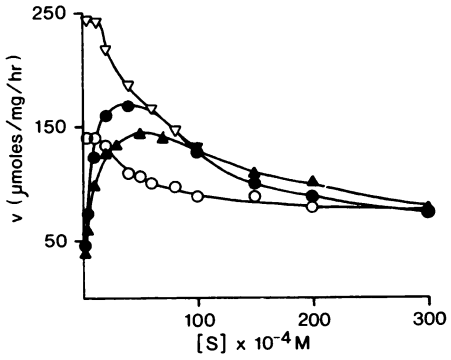

FIG. 10. *Effects of calcium, TEA, and gallamine on substrate inhibition of acetylcholinesterase.*

O——O, control (no added ions); △——△, 0.6 mM Ca^{++}; ▲——▲, 5 mM TEA; ●——●, 50 μM gallamine.

TABLE 2

Effect of combinations of TEA and TMA on acetylcholinesterase inhibition

Values for inhibition for TMA and TEA individually at the concentrations shown were determined at an acetylcholine concentration of 0.4 mM, in the absence of any other added ions. These individual inhibition values were used to calculate the inhibition expected for summation, by means of the formula $i_{1,2} = i_1 + i_2 - i_1 i_2$ (see the text). The two inhibitors were then combined after 5 min of incubation and their combined inhibition was obtained experimentally (third column).

TMA	TEA	Inhibition	
		Obtained	Calculated for summation
mM	*mM*	%	%
8.0	6.0	78.4	81.6
8.0	3.0	69.9	66.0
4.0	3.0	60.0	50.8
2.0	3.0	51.1	46.0
2.0	1.5	27.1	29.0

DISCUSSION

Kinetic data for the effects of Ca^{++}, TMA, TEA, and depolarizing and nondepolarizing neuromuscular blocking agents have been obtained, and the effects of small cations on the binding of neuromuscular blocking agents and substrate have been considered. The approach taken in the interpretation of data on the effects of combinations of two ligands considered any resulting antagonism to be due to competition for binding sites rather than to competition for two or more conformational states of the acetylcholinesterase subunit complex (13). This does not imply, however, that the specific effects due to ligand interactions with acetylcholinesterase (e.g., activation) are not due to specific conformational perturbations of the protein molecule (14).

The kinetic data for Ca^{++} (Fig. 1), at least at the lower activating concentrations, indicate that Ca^{++} does not compete with acetylcholine for the catalytic anionic site and hence must bind at an allosteric site, which we call the β-anionic site. The kinetics for Ca^{++} activation of acetylcholine hydrolysis differs from that for either Mg^{++} and Na^{+}; these ions inhibit substrate hydrolysis at low substrate concentrations (5, 15), are pure competitive inhibitors of a poor substrate (15), delay substrate inhibition, and activate acetylcholine hydrolysis only at higher substrate concentrations (5, 15). Mg^{++} and Na^{+} thus lack the specificity of Ca^{++}, since it appears that these ions can bind at the catalytic anionic site (α) as well as at the β-anionic site. Other workers have also suggested that inorganic ions bind to peripheral anionic sites (10–12). The β-anionic site proposed here would function as an accelerator site and bind relatively polar cations.

Decamethonium inhibition follows Michaelis-Menten kinetics (Fig. 3), and it is generally considered that decamethonium binds to the catalytic anionic site (α) and to a second allosteric site (1, 2). We have now shown that Ca^{++} antagonizes decamethonium binding in a competitive manner[3] (Fig. 4A and B), and this suggests that decamethonium binds across the α- and β-anionic sites, since antagonism by Ca^{++} presumably occurs at the β-site. TEA antagonizes decamethonium binding to the same degree as Ca^{++} (Fig. 7). Hence we conclude that TEA can also act at the β-anionic site. An allosteric binding site for TEA has also been suggested by others (8, 9). On the other hand, TEA is an inhibitor of acetylcholine at low substrate concentrations (5), is a pure competitive inhibitor of a poor substrate (7), and delays substrate inhibition (Fig.

[3] The antagonism of decamethonium by Ca^{++} observed in this study with erythrocyte acetylcholinesterase agrees with the finding of Changeux (1) for the enzyme purified from *Torpedo marmorata*, but not with results obtained by Wins *et al.* (11) on a membrane-bound preparation from the same source. These latter workers did not find antagonism between decamethonium and Ca^{++}; however, the concentrations of decamethonium used were 1000 times those required in the purified erythrocyte preparation used in this investigation and for the preparation used by Changeux (1). It is possible that in the membrane-bound preparation decamethonium did not bind to the enzyme in an analogous manner.

10). TEA must therefore have some affinity for the α-anionic site as well. TMA is a pure competitive inhibitor of acetylcholine hydrolysis (Table 1) and does not accelerate deacylation reactions (5, 6), and we now find that it is a relatively poor antagonist of decamethonium binding (Fig. 8). TMA must therefore have very little affinity for the β-anionic site, and probably acts at the α-catalytic site almost exclusively. Thus we propose that TEA and TMA may act at two different sites. This is supported by the fact that in combination they give additive inhibition (Table 2).

Neither TMA, TEA, nor Ca^{++} antagonizes the inhibition of acetylcholinesterase by gallamine. Since these cations most likely occupy the α- and β-sites, we propose that gallamine cannot bind to either the α- or β-anionic sites, and hence must bind at some other site, which we call the γ-anionic site. This may function as an accelerator site, preferentially binding hydrophobic molecules (3). It could correspond to the A_2 hydrophobic site proposed by Kabachnik *et al.* (16). Since gallamine does not bind to the β-anionic site, the antagonism of decamethonium by gallamine is probably due to antagonism between allosteric sites. The presence of gallamine at the γ-allosteric site may alter the kinetic pattern of activation by Ca^{++} and TEA at the β-site, and the inhibition by TMA at the α-anionic site.[4] Hence occupation of the γ-site can regulate activity of the catalytic and β-accelerator sites.

Our data (Fig. 6) showing some antagonism of *d*-tubocurarine inhibition by Ca^{++}, however, are compatible with the assumption that *d*-tubocurarine and decamethonium share a common anionic site, as proposed in the model of Belleau *et al.* (2). Thus *d*-tubocurarine could form a bridge between the β- and γ-anionic sites.

These observations have permitted us to expand on the model proposed by Belleau *et al.* (2). The expanded model is shown in Fig. 11. It is now widely accepted that acetylcholinesterase is a dimeric hybrid of two α- and two β-protein chains (13). Whether

[4] Unpublished observations.

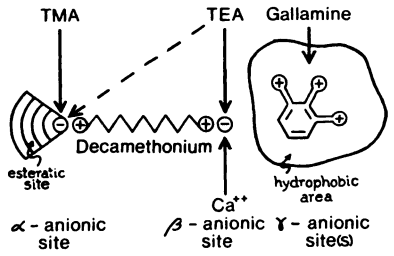

FIG. 11. *Proposed binding-site model for acetylcholinesterase.*

the anionic sites proposed are on the same or on different polypeptide chains, whether bifunctional inhibitors bridge the α-chains, the β-chains, or an α- and a β-chain, or perhaps even two tetramers, and the nature of the molecular changes induced by activators acting at allosteric sites remain to be determined by more sophisticated chemical analyses.

ACKNOWLEDGMENT

Excellent technical assistance in the latter stages of this work was provided by Mrs. Virginia Wickson.

REFERENCES

1. J.-P. Changeux, *Mol. Pharmacol.* **2**, 369 (1966).
2. B. Belleau, V. Di Tullio and Y.-H. Tsai, *Mol. Pharmacol.* **6**, 41 (1970).
3. R. J. Kitz, L. M. Braswell and S. Ginsberg, *Mol. Pharmacol.* **6**, 108 (1970).
4. J. L. Webb, "Enzyme and Metabolic Inhibitors," p. 157. Academic Press, New York, 1963.
5. B. D. Roufogalis and J. Thomas, *Mol. Pharmacol.* **4**, 181 (1968).
6. B. D. Roufogalis and J. Thomas, *Mol. Pharmacol.* **5**, 286 (1969).
7. B. D. Roufogalis and J. Thomas, *J. Pharm. Pharmacol.* **22**, 649 (1970).
8. R. D. O'Brien, *Biochem. J.* **113**, 713 (1969).
9. F. Iverson, *Mol. Pharmacol.* **7**, 129 (1971).
10. A. P. Brestkin, I. L. Brik, R. I. Volkova, E. B. Maizel' and E. V. Rozengart, *Biokhimiya* **35**, 382 (1970).
11. P. Wins, E. Schoffeniels and J. M. Foidart, *Life Sci.* **9**, Pt. 1, 259 (1970).
12. J. Gridelet, J. M. Foidart and P. Wins, *Arch. Int. Physiol. Biochem.* **78**, 259 (1970).
13. W. Leuzinger, *in* "Cholinergic Ligand Interactions" (D. J. Triggle, J. F. Moran and

E. A. Barnard, eds.), p. 19. Academic Press, New York, 1971.
14. I. B. Wilson, *in* "Cholinergic Ligand Interactions" (D. J. Triggle, J. F. Moran and E. A. Barnard, eds.), p. 1. Academic Press, New York, 1971.
15. B. D. Roufogalis and J. Thomas, *Life Sci.* **7**, Pt. 2, 985 (1968).
16. M. I. Kabachnik, A. P. Brestkin, N. N. Godovikov, M. J. Michelson, E. V. Rozengart and V. I. Rozengart, *Pharmacol. Rev.* **22**, 355 (1970).